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FILING DATE.

APPLICATION NUMBER: 60/431,897

FILING DATE: December 09, 2002

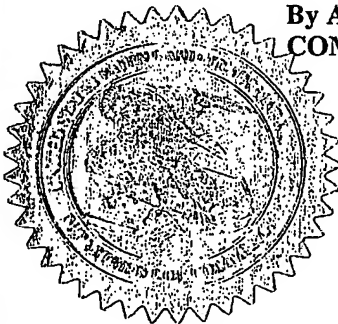
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12-10-021431737 12/09/02

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV 096 595 241 US

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TITLE OF THE INVENTION (500 characters max)

PEPTIDES AND METHODS FOR DEACTIVATION OF ORGANOPHOSPHORUS-BASED NERVE AGENTS AND INSECTICIDES

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification Number of Pages 26

☒ Drawing(s) Number of Sheets 5

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number _____

☒ Other (specify) Claims (6 pgs) Abstract (1 pg) Sequence Listing (3 pgs)

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Respectfully submitted,

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Date 12/09/2002

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60919-PRO/JPW/AJM/MML

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Dkt. 0575/60919-PRO/JPW/AJM/MML

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Donald W. Landry and Shi Xian Deng
Serial No.: Not Yet Known
Filed : Herewith
For : PEPTIDES AND METHODS FOR DEACTIVATION OF
ORGANOPHOSPHORUS-BASED NERVE AGENTS AND
INSECTICIDES

1185 Avenue of the Americas
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December 9, 2002

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PEPTIDES AND METHODS FOR DEACTIVATION OF ORGANOPHOSPHORUS-BASED NERVE AGENTS AND INSECTICIDES

The invention disclosed herein was made with United States
5 government support under grant numbers TLN 10-122 and DO
0788 from the Department of the Army, Army Research Office
1132; Scientific Services Program. Accordingly, the United
States government has certain rights in this invention.

10 Throughout this application, various publications are
referenced by author and date. Full citations for these
publications may be found at the end of the specification
immediately preceding the claims. The disclosures of these
references in their entireties are hereby incorporated by
15 reference into this application to describe more fully the
art to which this invention pertains.

Background of the Invention

Irreversible inhibitors of acetylcholinesterase (AChE) are
used extensively as insecticides and have also been used as
20 chemical weapons. World-wide stockpiles of these agents for
use in chemical warfare are estimated to exceed 200,000 tons
(Lejeune et al, 1998). The extreme toxicity of these
compounds and their global proliferation has made the
development of protective agents and antidotes an important
25 research priority.

AChE inhibitors are derivatives of phosphoric,
pyrophosphoric and phosphonic acids. These agents react to
form a phosphoester with the serine residue that resides in
30 the active site of the AChE enzyme. This phosphoester form

of the enzyme is inactive, although the active enzyme can be regenerated through hydrolysis of the phosphoester.

The toxicity of the organophosphorus agents arises from the stability of the phosphoester intermediate that is formed with the enzyme. As its name implies, AChE functions to cleave the ester linkage of acetylcholine, forming acetic acid and choline. To do this, the enzyme transiently forms an ester with the carboxyl moiety of acetylcholine, releasing choline. The resulting carboxylate ester of the enzyme is inactive. However, this ester undergoes rapid hydrolysis, measured in milliseconds, to regenerate the original, active form of the enzyme. In contrast, the rates of hydrolysis for the phosphate esters of AChE are measured in hours. The reversible inhibitors of AChE that are used clinically generate a carbamylated enzyme and have rates of hydrolysis measured in minutes. Thus, the time required for regeneration of the active enzyme via hydrolysis of the ester linkage of the inactive, acetylated enzyme defines the difference between the enzyme's normal function, reversible inhibition, and irreversible inhibition.

One strategy for developing antidotes to irreversible AChE inhibitors has been the synthesis of highly nucleophilic small molecules capable of efficiently cleaving phosphate esters. Hydroxylamine is one such compound that was demonstrated to significantly increase the rate of hydrolysis of phosphorylated AChE. However, efficient hydrolysis was only achieved at toxic concentrations of hydroxylamine.

To date, this approach has yielded only one compound that has shown clinical efficacy, 2-pyridine aldoxime

methyldichloride (2-PAM). The oxygen atom of this molecule is part of an oxime functional group. The oxime moiety is a hydroxylamine-like nucleophile formed from the reaction of hydroxylamine with aldehydes or ketones. The effectiveness of this compound is limited by its inability to cross the blood-brain barrier. However, effectiveness is further impaired by an inability to regenerate the enzyme once "aging" has occurred. This latter impediment means that treatment is only effective if administered within a few minutes to a few hours of toxin exposure, depending on the toxin.

Organophosphorus agents, particularly some of the more recent additions to chemical weapons arsenals, have the propensity to become truly irreversible inhibitors through the aging process. This molecular process occurs when, having first reacted with AChE to inactivate the enzyme, a phosphoester bond undergoes cleavage, resulting in an anionic ester which is extremely resistant to hydrolysis. The phosphorylated enzyme which has aged in this way is completely refractory to regeneration by currently available antidotal agents, including 2-PAM. It is this aging process that makes the phosphorus-derived chemical warfare agents, such as sarin, soman, VX and tabun, extremely lethal.

In addition to efforts to produce antidotes, research has focused on protecting against exposure to organophosphorus agents, thereby preventing or moderating their toxic effects. One important strategy has been the use of recombinant enzymes for the biocatalytic degradation of organophosphorus agents. This methodology is being utilized in the development of protective clothing as well as agents for surface or aerial decontamination. In these

methodologies, microbial enzymes, such as cholinesterases or organophosphorus hydrolases, are attached to a solid support in a manner that retains some portion of their catalytic activity. Such biocatalytic materials include silicone polymers (Gill 2000) and polyurethane foams (LeJeune 1996, 1999; Cheng 1996).

Peripheral blocking methods, i.e., methods that rely on agents that would intercept nerve agents in the circulatory system before they partition into the central nervous system or muscle, are currently the only choice for protection against organophosphorus agents. The standard technology in use today for protection against nerve agents is based upon the recombinant enzyme, butyrylcholinesterase (BuChE). BuChE rapidly reacts with nerve agents to form an intermediate phosphoester with a serine in the active site much as occurs when these agents react with AChE. Compounding the slow rate of hydrolysis of this intermediate is the inactivation of the enzyme through the process of molecular aging discussed above. Thus, BuChE is essentially an autocatalytic stoichiometric blocker rather than a true hydrolytic enzyme. Butyrylcholinesterase mutants demonstrate enzymatic turnover, but low turnover, combined with high immunogenicity, an inability to cross the blood-brain barrier and a high equivalent weight ratio (almost 500:1 for butyrylcholinesterase to nerve gas). These factors make it difficult to maintain *in vivo* enzyme levels sufficient to protect against a lethal dose of a toxin such as a nerve agent.

To date, the above-mentioned shortcomings have not been overcome.

This invention provides a method for determining whether a peptide forms a phosphorus-based ester with an organophosphorus agent. This invention also provides a method for determining whether, among a plurality of peptides, there exists a peptide that forms a phosphorus-based ester with an organophosphorus agent. This invention further provides a method for identifying and characterizing a peptide, among a plurality of peptides, that forms a phosphorus-based ester with an organophosphorus agent. This invention further provides a peptide which forms a phosphorus-based ester with an organophosphorus agent, which peptide comprises a nucleophilic functional group. This invention also provides a peptide library, wherein each peptide therein comprises a nucleophilic functional group. This invention further provides a composition of matter comprising a peptide and a pharmaceutical or a nonpharmaceutical carrier. This invention also provides an article of manufacture comprising a peptide affixed to a solid substrate.

This invention further provides a method for reducing the likelihood of injury due to exposure to an organophosphorus-containing agent in a subject exposed to or at risk of exposure to such agent, comprising administering to the subject an effective amount of a peptide of the instant invention.

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provides a kit for decontaminating an area exposed to an organophosphorus-containing agent comprising the instant peptides and instructions for use.

- 5 Finally, this invention provides a method for determining the presence of an organophosphorus-containing agent in an area.

Brief Description of the Figures

Figure 1: A sarin analog tethered to a fluorescent label was synthesized as shown to provide a low toxicity version of soman that would retain its chemical reactivity and report its reaction with a peptide molecule displayed on a library bead.

Figure 2: Structure of the fluorescently labeled sarin analog (1) and additional analogs with the following properties, hydrophobicity (2), water-solubility (3) and UV-activity (4).

Figure 3A: Analog (2) was synthesized by fluorination of 1-(isopropylhydroxyphosphinyl)-10-undecene with DAST.

Figure 3B: Synthesis of analog (3). The olefin (5) was oxidatively converted to aldehyde (6) with OsO_4 and NaIO_4 . Reductive amination of (6) with dimethyl amine and sodium borohydride afford the amine (7). Quaternization of the tertiary amine (7) with iodomethane in DMF gave (8) which was treated with DAST to afford the analog (3).

Figure 3C: Synthesis of analog (4). Treatment of phenylphosphonic dichloride with excess isopropanol in the presence of triethylamine afforded the diisopropyl phenylphosphonate which was subjected to basic hydrolysis with NaOH to generate the monoester (isopropyl phenylphosphonic acid). The nerve gas analog (4) was generated by treatment with DAST in a manner similar to analog (1).

Detailed Description of the Invention

Definitions

In this invention, "administering" can be effected or performed using any of the various methods and delivery systems known to those skilled in the art. The administering can be performed, for example, intravenously, orally, via implant, transmucosally, transdermally, intramuscularly, and subcutaneously. In a preferred embodiment, the peptide is administered intramuscularly.

Determining an "effective amount" of the instant peptide for decontamination purposes can be done based on *in vitro* data. Determining an effective amount of the instant peptide for administering to a subject can be done based on animal data using routine computational methods. In one embodiment, the effective amount contains between about 10 mg and 1000 mg of the instant peptide. In another embodiment, the effective amount contains between about 50 mg and about 500 mg of the peptide. In a further embodiment, the effective amount contains between about 100 mg and about 250 mg of the peptide, and preferably about 200 mg thereof.

"Pharmaceutical carriers" are well known in the art and include, but are not limited to, 0.01-0.1 molar phosphate buffer, 0.8% saline solution, propylene glycol, polyethylene glycol, vegetable oils and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed

oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

"Nonpharmaceutical carriers" include, without limitation, foams and aerosols. For example, a polyurethane foam can be synthesized to incorporate a peptide of the instant invention using methods standard in the art of polymer synthesis. In one example, a hydrophilic polyurethane prepolymer is reacted with water containing the peptide and a surfactant, forming a foam. Such prepolymers are known in the art and comprise a urethane capped with multiple isocyanate functionalities. As one of skill in the art would be aware, various surfactants can be used in order to alter the porosity, density and surface properties of the foam.

The term "organophosphorus agent" is synonymous with "organophosphorus-based agent", and is used herein to refer to a chemical compound comprising carbon, hydrogen, phosphorous and oxygen. Organophosphorus agents include, without limitation, phosphorus-based esters. A number of commercially important insecticides, as well as chemical warfare agents, contain a phosphorus-based ester moiety which either is inherently toxic to humans or is readily converted into a toxic moiety. Examples of such agents include, without limitation, malathion, parathion, paraoxon, schradan, dichlorfenthion, soman, sarin, VX, GB and tabun.

The term "phosphorus-based ester" is used herein to refer to any ester of phosphorous. Examples of such esters include,

without limitation, a phosphate ester, a phosphonate ester and a phosphinate ester.

The term "peptide" is used herein to refer to a polymer of amino acid residues. In one embodiment, a peptide has 15 or fewer amino acid residues. Amino acid residues include, without limitation, any of the 20 naturally occurring amino acids or modifications thereof, as well as natural or synthetic derivatives thereof. A peptide can also comprise one or more modified amino acid residues. Modifications include, without limitation, the conjugation to an amino acid residue of a non-protein moiety (e.g. an oxime or a cyclodextran). Other modifications include the addition of a chemical tag useful for identification or purification. Examples of chemical tags include, without limitation, a biotin molecule, a radioisotope and a fluorescent molecule.

As used herein, a "solid substrate" and a "solid support" are synonymous, and shall mean any water- and/or lipid-insoluble support to which a peptide can be affixed. Examples of solid supports include, without limitation, alumina pellets, trityl agarose and glass/silica beads, and polymers such as nylons, acrylates, and silicas.

"Subject" shall mean any animal, such as a primate, mouse, rat, guinea pig or rabbit. In the preferred embodiment, the subject is a human

Embodiments of the Invention

This invention provides a method for determining whether a peptide forms a phosphorus-based ester with an organophosphorus agent comprising the steps of contacting the peptide with the agent under conditions permitting the

formation of a phosphorus-based ester and determining whether a phosphorus-based ester has formed.

5 This invention also provides a method for determining whether, among a plurality of peptides, there exists a peptide that forms a phosphorus-based ester with an organophosphorus agent. This method comprises the steps of contacting the plurality of peptides with the agent under conditions permitting formation of a phosphorus-based ester
10 and determining whether a phosphorus-based ester has formed.

15 This invention further provides a method for identifying and characterizing a peptide among a plurality of peptides that forms a phosphorus-based ester with an organophosphorus agent. This method comprises the steps of contacting the agent with the plurality of peptides under conditions permitting the formation of a phosphorus-based ester, identifying the peptide or peptides that form such an ester with the agent and determining the amino acid sequence of
20 the peptide so identified.

In an embodiment of any of these methods, the organophosphorus agent which reacts with a peptide is selected from the group consisting of malathion, parathion,
25 paraoxon, schradan, dichlorofenthion, soman, sarin, VX, GB and tabun, or an analog thereof.

In any of these three methods, the phosphorus-based ester can be any ester of phosphorus including, for example, a
30 phosphate ester, a phosphonate ester or a phosphinate ester.

In one embodiment of the instant methods, the peptides are bound to a solid support such as a bead, a microtiterplate,

a glass chip or a silicone chip. In another embodiment, the agent is labeled with a detectable marker. In a preferred embodiment, the detectable marker is a radioisotope, a fluorescent molecule, biotin or an enzyme. In one example, the agent is a nerve agent and the detectable marker is rhodamine. In another example, the agent has the structure set forth as analog (1) in Figure 2, which is a rhodamine-conjugated analog of the nerve agent soman.

- 10 In another embodiment of the instant methods, the peptides are not bound to a solid support. Instead, the agent or peptide is labeled with a moiety that provides a basis for affinity purification. Such moieties are well-known in the art and include, for example, biotin and glutathione. Such
- 15 purification can comprise a step of removing unreacted peptide and agent prior to affinity purification. This step may be accomplished in a number of ways using techniques that are well-known in the art, such as chemical separation based on solubility, size exclusion chromatography and gel
- 20 electrophoresis.

This invention also provides a peptide which forms a phosphorus-based ester with an organophosphorus agent, which peptide comprises a nucleophilic functional group. In one embodiment of this invention, the nucleophilic functional group comprises a thiol or a hydroxyl group. In a preferred embodiment, the peptide is between six and 15 amino acids in length or has a molecular weight of less than 1500 daltons. In one embodiment, the peptide is six amino acids in length.

In the preferred embodiment of this invention, the agent with which the peptide reacts is an organophosphorus insecticide or chemical warfare agent.

This invention also provides a peptide library, wherein each peptide therein comprises a nucleophilic functional group. In one embodiment, each peptide in the library comprises a
 5 thiol or hydroxyl-containing amino acid residue, and the position at which such thiol or hydroxyl-containing amino acid residue occurs is the same for each peptide in the library. In another embodiment, each peptide is of a fixed length. In a further embodiment, the length of each peptide
 10 is between six and 15 amino acid residues and/or the molecular weight of each peptide is less than 1500 daltons. In a further embodiment, each peptide has a length of six amino acid residues. In still another embodiment, the first, second, third, fourth, fifth, or sixth amino acid residue in
 15 each hexapeptide is a serine.

This invention also provides a composition of matter comprising one of the instant peptides and a pharmaceutical carrier. This invention further provides a composition of
 20 matter comprising one of the instant peptides and a nonpharmaceutical carrier. In one embodiment, the nonpharmaceutical carrier is a foam or aerosol.

Foam formulations and mechanisms for their dispersal are
 25 well-known in the art and are particularly useful for surface decontamination. Foam formulations specifically designed for blast suppression are also known in the art and would be particularly useful in combination with the peptides of the instant invention in containing an explosive
 30 device that has been designed to disseminate a nerve agent.

This invention also provides an article of manufacture comprising one of the instant peptides affixed to a solid

substrate. In one embodiment, the solid substrate is a polymer. In another embodiment, the solid substrate is a fabric or fiber. In yet another embodiment, the solid substrate is a filtration component, such as one used in a gas mask.

In the preferred embodiment, the peptides of the instant invention are incorporated into polymers using art-recognized techniques. Many different types of polymers are known in the art. The choice of polymer would depend, for example, upon whether the peptide is to be incorporated into protective clothing, a device such as a gas mask or a pellet or sheet for surface decontamination.

This invention provides a method for reducing the likelihood of injury due to exposure to an organophosphorus agent in a subject exposed to or at risk of exposure to such an agent. This method comprises administering to the subject an effective amount of a peptide of the instant invention.

This invention also provides a method for decontaminating an area exposed to an organophosphorus agent comprising introducing to the area an effective amount of a peptide of the instant invention.

This invention further provides a kit for decontaminating an area exposed to an organophosphorus agent comprising a peptide of the instant invention and instructions for use.

Finally, this invention provides a method for determining the presence of an organophosphorus agent in an area. This method comprises the steps of contacting a peptide of the instant invention with a sample taken from the area and

determining whether a phosphorus-based ester is formed with the peptide. In this method, the formation of such an ester is indicative of the presence of an organophosphorus agent in the area.

5

This invention is illustrated in the Experimental Details section which follows. This section is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the
10 invention as set forth in the claims which follow thereafter.

Experimental Details

Synopsis

The present invention provides methods and reagents for protection against poisoning by organophosphorus-based insecticides and nerve agents. In a novel approach to producing such reagents, this invention utilizes combinatorial chemistry and high-throughput screening to generate catalytic peptides for the inactivation of organophosphorus-based toxins. The peptides provided by the instant invention are selected based on their ability to autocatalytically bind and thereby inactivate substrate phosphoesters and offer several advantages over current enzyme-based approaches. These include decreased immunogenicity, and more favorable equivalent weight ratios.

Methods

Design of the peptide library

A combinatorial approach based on "split and pool" synthesis was used to generate a diverse population of peptides. Hexapeptide libraries were designed with five random positions and one fixed serine, giving 3.2 million unique peptides per library. Separate libraries were generated that contained a serine at each position, $[x_1, x_2 \dots x_6]$ in the peptide. Thus, a total of six libraries is produced, each having serine fixed in a different position of the hexapeptide.

Synthesis of the peptide libraries

Tental GelTM was used as the solid phase support and sequences of active peptides were determined using mass

spectrometry (MS). Peptide library synthesis was accomplished by a "split and pool" methodology using standard Fmoc peptide chemistry. For example, the first library, in which serine occupies the first position, x_1 , was synthesized as follows: (i) the beads were reacted with Fmoc-protected serine using benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and hydroxybenzotriazole (HoBt) as coupling agents, (ii) amino groups were blocked with acetic anhydride, and (iii) the Fmoc protecting group was removed using 20% piperidine in DMF. The next five amino acids in the peptide were placed randomly using the "split and pool" method. This is accomplished by separating the entire population of beads into twenty groups of equal size. Each of the twenty amino acids is reacted with an individual group using BOP and HoBt as coupling agents. After the coupling reaction, the beads are combined so that they can be washed, blocked, and finally deprotected together. This split and pool synthesis is performed five times in order to add five random amino acids to each peptide.

Library screening

Libraries were exposed to a fluorescently labeled nerve gas analog for 1 hr followed by 1 minute washes at room temperature in PBS containing 10% Tween 20, 10% DMF, followed by DMF containing 10% Tween 20. Butylcholinesterase coupled to native beads was used as a positive control. Library peptides which remained fully protected and thus unreactive were used as a negative control. Wash conditions were optimized to exclude noncovalent interactions between the fluorescent label and the beads utilizing either peptides in which the reactive groups were blocked by pre-incubation with either acetic anhydride or a single "non-

specific" peptide not likely not to be configured for reaction.

Following incubation with analog (1) and extensive washing, beads displaying relatively high levels of fluorescence were selected and their peptides sequenced using LCMS. Peptides corresponding to those identified in the screen were synthesized using Tentel Gel™ as the solid phase and standard Fmoc chemistry, then purified using high-pressure liquid chromatography (HPLC).

Synthesis of fluorescent sarin analogs

Fluorescent-labeled sarin analogs were synthesized as shown in Fig. 1. Briefly, 10-undecen-1-ol (1) was converted to an iodinated compound (3) through a tosylate intermediate (2). Reaction of (3) with excess tri-isopropylphosphite under reflux conditions afforded the di-isopropoxy phosphonate (4), which was converted to the isopropoxyhydroxy phosphonate (5) by refluxing with NaOH in methanol. The olefin (5) was oxidatively cleaved with ruthenium trichloride and sodium periodate to yield the terminal carboxylic acid (6). Treatment of (6) with excess diethylaminosulfur trifluoride (DAST), followed by N-hydroxysuccinimide (NHS), afforded the N-succinyl fluorophosphonate intermediate (8). Without further purification compound (8) was reacted with amine-functionalized rhodamine (NH₂-rhodamine) to generate the rhodamine-labeled sarin analog (9). The structures of the compounds 2-8 and 9 (analog 1) were determined by NMR and MS. Experimental details are set forth below.

Compound (2) [1-((p-toluenesulfonyl)oxy)-10-undecene]: A solution of (1) (5 g, 0.029 mol) in pyridine (50 ml) was

reacted with *p*-toluenesulfonyl chloride (8.2 g, 0.044 mol) at room temperature for 4 h. The reaction mixture was evaporated and the residue was dissolved in ethyl acetate. The acetate solution was washed with 1N HCl (2x30ml) and concentrated under reduced pressure. The product (2) was purified by chromatography (SiO₂, ethyl acetate/hexane 1/20). Colorless oil, yield 8.9 g, 94%, ¹H NMR (CDCl₃, 300MHz): 7.7(d, 2H), 7.32(d, 2H), 5.8(m, 1H), 5.0(m, 2H), 4.0(t, 2H), 2.4(s, 3H), 2.0(m, 2H), 1.7(m, 2H), 1.5-1.2(m, 12H); MS: found 324.18, calcd. 324.18 for C₁₈H₂₈O₃S.

Compound (3) [1-iodo-10-undecene]: A solution of (2) (8.0 g, 0.025 mol) in acetone (80 ml) was treated with NaI (7.0 g, 0.05 mol). The reaction mixture was refluxed for 2 h and concentrated. The residue was dissolved in ethyl acetate (50 ml) and washed with H₂O (20 ml). The product (3) was purified by chromatography (SiO₂, ethyl acetate/hexane 1/20). 5.5 g oil, 79%. ¹H NMR (CDCl₃, 300 MHz): 5.8(m, 1H), 5.0(m, 2H), 3.2(t, 2H), 2.0(m, 2H), 1.8 (m, 2H), 1.50-1.20(m, 12H). MS: found 280.07, calcd. 280.07 for C₁₁H₂₁I.

Compound (4) [1-(di-isopropoxy) phosphinyl]-10-undecene]: A mixture of triisopropyl phosphite (10 ml, excess) and compound (3) (5.0 g) was refluxed for 15 h. Excess of triisopropyl phosphite was removed by distillation under reduced pressure. The product was purified by chromatography (SiO₂, ethyl acetate/hexane 1/20). Oil, 5.4 g, 93%. ¹H NMR (CDCl₃, 300 MHz) 5.70 (m, 1H), 4.9 (m, 2H), 4.80 (m, 2H, CHOP), 2.0-1.0 (m, 30H). MS: found 318.380, calcd. 318.380 for C₁₇H₃₅O₃P.

Compound (5) [1-(isopropylhydroxyphosphinyl)-10-undecene]: A solution of compound (4) (5.0 g, 0.016 mol) in methanol (80

ml) and aqueous NaOH (5.0 g in 15 ml H₂O) was refluxed for 5 h. The solvents were removed by concentration under reduced pressure. The residue was dissolved in H₂O (30 ml) and extracted with ethyl ether. The aqueous solution was
5 acidified with 6 N HCl to pH=2, and extracted with ethyl acetate. The organic phase was evaporated to give pure compound (5) as clear oil. 3.9g, 89%. ¹H NMR (CDCl₃, 300 MHz) 5.70 (m, 1H), 4.9 (m, 2H), 4.80(m, 1H, CHOP), 2.0-1.0 (m, 24H).

10

Compound (6) [10-(iso-propylhydroxyphosphinyl) decanoic acid]: To compound (5) (1.0g, 3.6 mmol) dissolved in CCl₄-CH₃CN-H₂O (1:1:1.5) (35 ml) was added NaIO₄ (3.1g, 4 eq.) and ruthenium trichloride hydrate (20 mg). The reaction mixture
15 was stirred for 2 h at room temperature and then partitioned between CH₂Cl₂ (40 ml) and 1N HCl (20 ml). The organic layer was concentrated under reduced pressure and the resulting residue was purified by chromatography to give semisolid product (SiO₂, ethyl acetate/methanol ½). 0.7 g, 70%. ¹H NMR
20 (CDCl₃) 4.70 (m, 1H, CHOP), 2.3 (t, 2H), 2.0-1.0 (m, 24H). High resolution MS: found 295.166, calcd. 295.166 for C₁₃H₂₈O₅P.

Compound (9) [Rhodamine-labeled sarin analog (1)]: A
25 solution of (6) (100 mg, 0.34 mmol), in CH₂Cl₂ (3 ml) was treated at -78°C with diethylaminosulfur trifluoride (DAST, 0.1 mL, 0.5 mmol), and the reaction mixture was stirred for 10 min. The resulting difluoride (7) was treated *in situ* with N-hydroxysuccinimide (0.4 mmol) in DMF (0.2 ml) and
30 stirred for another 5 minutes at room temperature. The reaction mixture was partitioned between ethyl acetate (15 ml) and water (10 ml), and the organic layer was washed with brine (5 ml), dried with Na₂SO₄ and concentrated under

reduced pressure to afford the N-hydroxysuccinimide (8). Without further purification, this compound was treated with amine-functionalized rhodamine derivative (1.0 eq) in DMF (2 ml) and stirred for 20 minutes. The solvents were evaporated under reduced pressure. The remaining residue was washed sequentially with diethyl ether and ethyl acetate to afford (9) as a red film. 150 mg, 49%. ¹H NMR (CDCl₃, 300 MHz) 7.0-8.0 (m, 9H), 4.90 (m, 1H, CHOP), 2.5-1.0 (m, 46H). High resolution MS: found 793.4105, calcd. 793.4400 for C₄₃FH₅₉N₄O₇P.

Synthesis of additional sarin analogs

Synthesis of analog (2): Analog (2) was synthesized by fluorination of compound (5) (Figure 1), 1-(isopropylhydroxyphosphinyl)-10-undecene, with DAST in a similar manner to that of analog (1) shown in Figure 1 and described above. The synthetic scheme of analog (2) is summarized in Figure 3A.

Synthesis of analog (3): Analog (3) was synthesized by the route outlined in Scheme 2, Figure 3B. The olefin (5) was oxidatively converted to an aldehyde (6) with OsO₄ and NaIO₄. Reductive amination of (6) with dimethyl amine and sodium borohydride afforded the amine (7). Quaternization of the tertiary amine (7) with iodomethane in DMF gave (8) which was treated with DAST to afford the analog (3).

Experimental details for analog (3): Compound (5) (0.74 g) in a solution of THF (10 ml) H₂O (6 ml) was added to 2% OsO₄ in H₂O (2 ml) and NaIO₄ (2.0 g, 4 eq.). The mixture was stirred at room temperature for 18 h, quenched with saturated Na₂SO₃ (10 ml) and partitioned with ethyl acetate. Evaporation of the solvents afforded the aldehyde (6) [oil,

0.5 g. 70% by NMR]. The aldehyde (0.45 g) (6) in ethanol (50 ml) was added to dimethylamine (1.3 eq). To this solution was added NaBH_4 (1.1 eq). The reaction mixture was stirred for 4 h and the treated with 1 N HCl at 0°C. After
 5 removal of the solvents, the residue was dissolved in water and washed with ethyl ether. The aqueous layer was concentrated under reduced pressure to afford compound (7) [0.32 g, 63% by NMR]. The amine (7) (0.3 g) in DMF (10 ml) was treated with MeI (1 ml, excess) overnight. The solvent
 10 was removed under reduced pressure to give compound (8) which was converted to analog (3) in a similar manner as analog (1) [semisolid, 85% by NMR].

Synthesis of analog (4): Analog (4) was synthesized by the
 15 route outlined in scheme 3, Figure 3C. Treatment of phenylphosphonic dichloride with excess isopropanol in the presence of triethylamine afforded the diisopropyl phenylphosphonate which was subjected to basic hydrolysis with NaOH to generate the monoester (isopropyl
 20 Phenylphosphonic acid). The nerve gas analog (4) was generated by treatment with DAST in a manner similar to analog (1).

Solution-phase reaction of peptides with sarin analogs

1 mmol of purified peptide was incubated with one of the
 25 nerve agent analogs (1.2 mmol) in PBS at room temperature for 2-3 hr. The formation of reaction product was monitored by HPLC (C_{18} analytic column, gradient eluting with 100% H_2O -70% MeCN for 1 h) and confirmed by mass spectrophotometry.

Results and Discussion

30 In order to obtain catalytic peptides with the desired reactivity, libraries consisting of peptides containing a

nucleophilic moiety were conceived which would react with the phosphonate moiety of organophosphorus-based agents to form a peptide phosphoester.

- 5 Initial work was done using hexapeptide libraries consisting of a serine at a fixed position $[x_1, x_2, \dots, x_6]$ and any amino acid at each of the remaining five positions. In one example, the hydroxyl moiety of serine serves as the nucleophile which reacts with the phosphonate of the agent to form a peptide-phosphoester, effectively blocking the ability of the agent to react with cellular proteins and thus neutralizing it.

- Each of the six hexapeptide libraries consists of at least 3.2 million peptides comprised of a random amino acid at each of five positions and a serine at a fixed position (given by probability $20^5 = 3.2 \times 10^6$). For example, Library 1 consists of at least 3.2 million peptides, each having a serine in position one (x_1) and any amino acid in each of the remaining five positions. In practice, each peptide was represented by approximately 6 copies in the library. Initially, three libraries were synthesized containing a fixed serine at either x_1 , x_2 , or x_3 , respectively.

- 25 In order to identify peptides with the desired reactivity, labeled analogs of the nerve gas sarin were synthesized. Labeled butylcholinesterase (BuChE) coupled to the same solid support as the library (in this case Tentel Gel™) was used as a positive control to identify peptides with suitable reaction kinetics.

Although radiolabeled analogs are preferable because they minimize interference due to steric hinderance, such analogs

5 beads as negative controls demonstrated that rhodamine-
conjugated sarin analog (1) produced the optimal signal to
noise ratio.

Library screening was performed with analog (1) and
10 fluorescent beads were selected. Initially, beads
demonstrating the highest fluorescence intensity were
manually selected under the microscope. However, this
technique could easily be optimized for selection using
high-throughput methods such as flow cytometry (for
15 libraries coupled to beads), plate readers (for libraries
adsorbed onto microtiter plates) or microarray scanners (for
libraries on microchips).

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manually selected under the microscope. However, this
technique could easily be optimized for selection using
high-throughput methods such as flow cytometry (for
15 libraries coupled to beads), plate readers (for libraries
adsorbed onto microtiter plates) or microarray scanners (for
libraries on microchips).

23 of the manually selected fluorescent beads were chosen
20 for sequencing and a further subset of ten of the resulting
peptides was chosen for resynthesis and further study. These
ten peptides had the following sequence: YKDNSY (SEQ ID
NO:1), YKDISY (SEQ ID NO:2), DNEFSY (SEQ ID NO:3), ANKYSY
(SEQ ID NO:4), YYCDSY (SEQ ID NO:5), YHYYSY (SEQ ID NO:6),
25 ANYYSY (SEQ ID NO:7), YEYQSY (SEQ ID NO:8), LIFASY (SEQ ID
NO:9), YKEFSY (SEQ ID NO:10), and CAYCSY (SEQ ID NO:11).

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disappeared at retention time 17 min, and the 3 new peaks appeared at 23, 25 and 29 min. ^1H NMR analysis demonstrated that the peaks at 4.9 ppm (H for $\text{Me}_2\text{CHP}(\text{O})\text{F}$) shifted to 4.5 ppm (H for $\text{Me}_2\text{CHP}(\text{O})\text{O}$), indicating formation of a
 5 phosphoester with the sarin analog. Diastereomers likely account for two of the products.

References

- Cheng, T.C. et al., Cloning and expression of a gene encoding a bacterial enzyme for decontamination of organophosphorus nerve agents and nucleotide sequence of the enzyme. *Appl. Env. Microbiol.* 62, 1636-1641 (1996).
- 5 Doctor, B.P., Richard K., et al., Immobilized enzymes: Nerve agent detoxifiers. *Proceedings of the ERDEC Scientific Conference on Chemical and Biological Defense Research, Aberdeen Proving Ground, MD, United States, Nov. 17-20, 1998, p. 559-566 (1999).*
- 10 Gill, I., and Ballesteros, A., Degradation of organophosphorous nerve agents by enzyme-polymer nanocomposites: efficient biocatalytic materials for personal protection and large-scale detoxification. *Biotech. Bioeng.* 70(4), 400-410 (2000).
- 15 LeJeune, K.E., Wild, J.R., Russell, A.J., Nerve agents degraded by enzymic foams. *Nature* 395(6697), 27-28 (1998).
- 20 Lejeune, K.E., Dravis, B.C., Yang, F., Hetro, A.D., Doctor, B.P., Russell, A.J., Fighting nerve agent chemical weapons with enzyme technology. *Ann. N.Y. Acad. Sci.* 864, 153-170 (1998).
- 25 LeJeune, K.E. and Russell, A.J., Biocatalytic nerve agent detoxification in fire fighting foams. *Biotechnol. Bioeng.* 62(6), 659-665 (1999).
- 30

What is claimed is:

1. A method for determining whether a peptide forms a
5 phosphorus-based ester with an organophosphorus agent
comprising the steps of:
 - (a) contacting the peptide with the agent under
conditions permitting formation of a phosphorus-
based ester; and
 - 10 (b) determining whether a phosphorus-based ester has
formed between the peptide and the agent.
2. A method for determining whether, among a plurality of
15 peptides, there exists a peptide that forms a
phosphorus-based ester with an organophosphorus agent
comprising the steps of:
 - (a) contacting the plurality of peptides with the
agent under conditions permitting formation of a
phosphorus-based ester; and
 - 20 (b) determining whether a phosphorus-based ester has
formed between any of the peptides and the
agent, the formation of such an ester indicating
that there exists a peptide that forms a
phosphorus-based ester with the agent.
- 25 3. A method for identifying and characterizing a peptide,
among a plurality of peptides, that forms a phosphorus-
based ester with an organophosphorus agent comprising
the steps of:
 - 30 (a) contacting the agent with the plurality of
peptides under conditions permitting the
formation of a phosphorus-based ester;

- (b) identifying a peptide among the plurality of peptides that forms a phosphorus-based ester with the agent; and
 - (c) determining the amino acid sequence of the peptide so identified in step (b), thereby identifying and characterizing the peptide that forms a phosphorus-based ester with the agent.
4. The method of claim 1, 2 or 3, wherein the phosphorus-based ester is a phosphate ester.
5. The method of claim 1, 2 or 3, wherein the phosphorus-based ester is a phosphonate ester.
6. The method of claim 1, 2 or 3, wherein the phosphorus-based ester is a phosphinate ester.
7. The method of claim 1, 2 or 3, wherein the peptides are bound to a solid support.
8. The method of claim 7, wherein the solid support comprises a bead, a microtiter plate, a glass chip or a silicone chip.
9. The method of claim 1, 2, or 3, wherein the agent is selected from the group consisting of malathion, parathion, paraoxon, schradan, dichlorfenthion, soman, sarin, VX, GB and tabun.
10. The method of claim 9, wherein the agent is labeled with a detectable marker.

11. The method of claim 10, wherein the detectable marker is a radioisotope, a fluorescent molecule, biotin or an enzyme.
- 5 12. The method of claim 11, wherein the agent is a nerve agent and the detectable marker is rhodamine.
13. The method of claim 12, wherein the agent has the structure set forth as analog 1 in Figure 2.
- 10 14. A peptide which forms a phosphorus-based ester with an organophosphorus agent, which peptide comprises a nucleophilic functional group.
- 15 15. The peptide of claim 14, wherein the functional group is a thiol or a hydroxyl group.
16. The peptide of claim 14, wherein the length of the peptide is between six and 15 amino acid residues.
- 20 17. The peptide of claim 16, wherein the peptide has a length of six amino acid residues.
18. The peptide of claim 14, wherein the molecular weight of the peptide is less than 1500 daltons.
- 25 19. The peptide of claim 14, wherein the agent is an organophosphorus insecticide or chemical warfare agent.
- 30 20. The peptide of claim 19, wherein the agent is selected from the group consisting of malathion, parathion, paraoxon, schradan, dichlorfenthion, soman, sarin, VX, GB and tabun.

21. A peptide library, wherein each peptide therein comprises a nucleophilic functional group.
- 5 22. The library of claim 21, wherein the nucleophilic functional group is a thiol or a hydroxyl group.
23. The library of claim 22, wherein each peptide in the library comprises a thiol or hydroxyl-containing amino
10 acid residue, and the position at which such thiol or hydroxyl-containing amino acid residue occurs is the same for each peptide in the library.
24. The library of claim 23, wherein each peptide of the
15 library is of a fixed length.
25. The library of claim 24, wherein the length of each peptide is between six and 15 amino acid residues and/or the molecular weight of each peptide is less
20 than 1500 daltons.
26. The library of claim 25, wherein each peptide has a length of six amino acids.
- 25 27. The library of claim 26, wherein the first, second, third, fourth, fifth or sixth amino acid residue in each hexapeptide is a serine.
28. A composition of matter comprising the peptide of claim
30 14, and a pharmaceutical carrier.
29. A composition of matter comprising the peptide of claim 14, and a nonpharmaceutical carrier.

30. The composition of claim 29, wherein the nonpharmaceutical carrier is a foam or an aerosol.
- 5 31. An article of manufacture comprising the peptide of claim 14 affixed to a solid substrate.
32. The article of claim 31, wherein the solid substrate is a polymer.
- 10 33. The article of claim 31, wherein the solid substrate is a fabric or fiber.
34. The article of claim 31, wherein the solid substrate is a filtration component of a gas mask.
- 15 35. A method for reducing the likelihood of injury due to exposure to an organophosphorus agent in a subject exposed to or at risk of exposure to such agent, comprising administering to the subject an effective amount of the peptide of claim 14.
- 20 36. A method for decontaminating an area exposed to an organophosphorus agent comprising introducing to the area an effective amount of the peptide of claim 14.
- 25 37. A kit for decontaminating an area exposed to an organophosphorus agent comprising the peptide of claim 14 and instructions for use.
- 30 38. A method for determining the presence of an organophosphorus agent in an area comprising the steps of:

- (a) contacting the peptide of claim 14 with a sample taken from the area; and
- (b) determining whether a phosphorus-based ester is formed with the peptide in step (a), the formation of such ester indicating the presence of an organophosphorus agent in the area.

PEPTIDES AND METHODS FOR DEACTIVATION OF ORGANOPHOSPHORUS-
BASED NERVE AGENTS AND INSECTICIDES

Abstract of the Disclosure

5 This invention provides methods and peptides for the
inactivation of organophosphorus-based insecticides and
chemical warfare agents. The instant methods include peptide
screening methods, peptides and peptide libraries, related
compositions of matter, articles of manufacture, and methods
for prophylaxis, treatment, decontamination and detection.

Figure 1

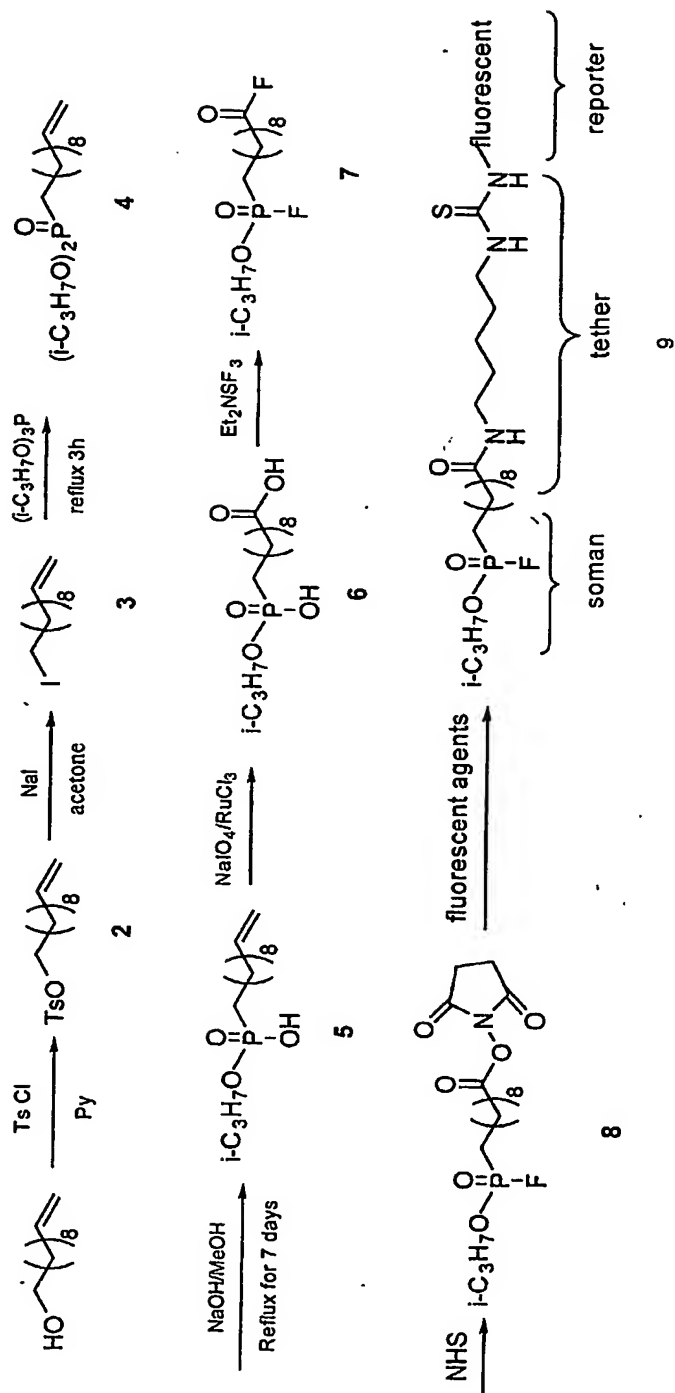


Figure 2

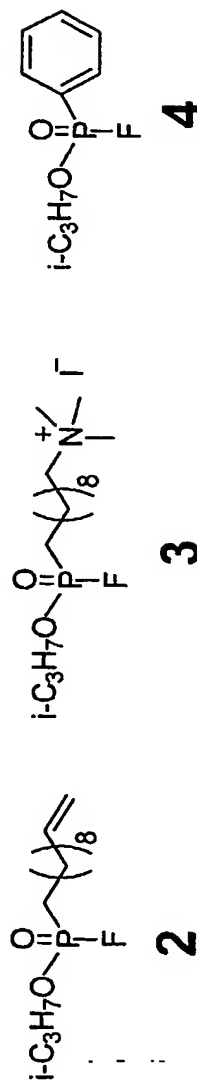
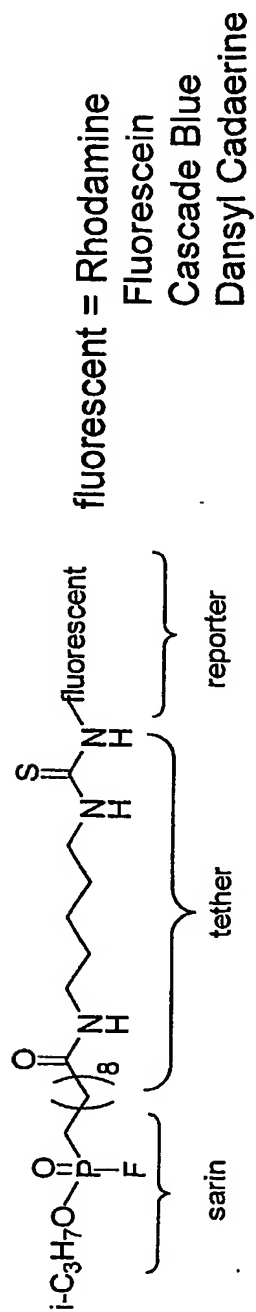


Figure 3A

Scheme 1



Analog 2

Figure 3B

Scheme 2

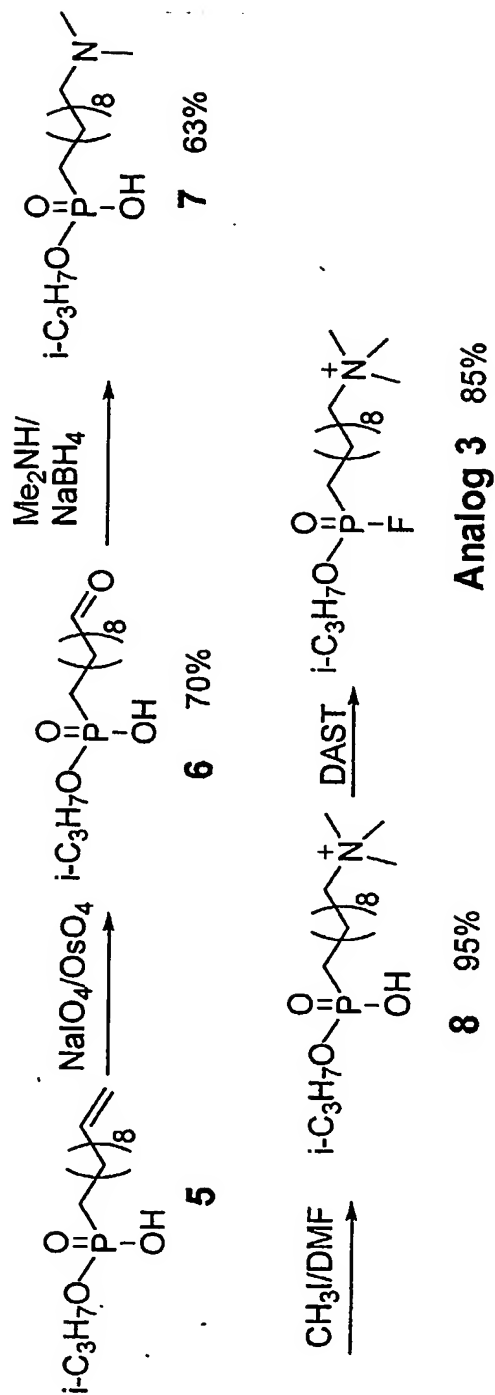
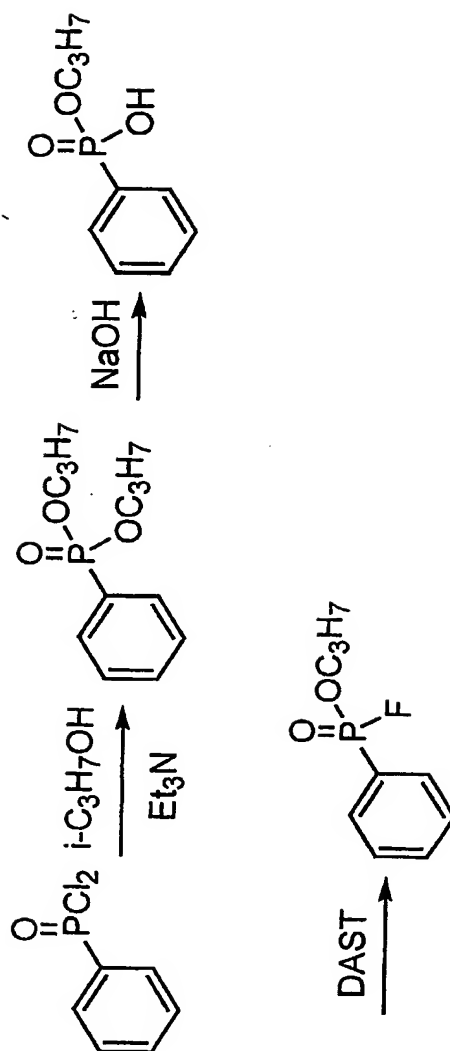


Figure 3C

Scheme 3



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